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(54) **Molecular cloning and expression in industrial microorganism species.**

(57) Novel methods and novel industrial unicellular microorganism strains, particularly industrial Bacillus strains, are provided for enhanced production of endogenous and exogenous polypeptides. Cloning vehicles containing one or more copies of the gene expressing the polypeptide of interest are introduced into a compatible host, the transformed hosts treated to form protoplasts and then fused with protoplast acceptors prepared from industrial strains. Efficient transfer of the vehicle containing the genes of interest is achieved, with the resulting modified industrial strains being effective producers of the desired polypeptide product.

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MOLECULAR CLONING AND EXPRESSION IN INDUSTRIAL MICROORGANISM SPECIES

BACKGROUND OF THE INVENTION

Field of the Invention

There is substantial interest in employing industrial unicellular microorganism strains as hosts with recombinant DNA to produce polypeptides in high yields. Many industrially important enzymes, such as amylolytic and proteolytic enzymes, are produced by microorganisms of the genus Bacillus, e.g., B. subtilis, B. amyloliquefaciens, B. licheniformis, B. stearothermophilus and B. coagulans. In fermenters, strains are employed which are highly robust and stable. Furthermore, the strains are resistant to phage infection and, in addition, to genetic exchange, that is introduction of DNA by conventional transformation procedures. The conventional industrial strains are also prototrophic, in order to avoid adding expensive amino acids to the nutrient medium. Other characteristics of industrial strains are their high productivity until the end of the fermentation, which can be as long as a week, stable cell concentration upon exhaustion of the broth, and high productivity, usually at least about 0.5% w/v secreted protein. In addition, it is often found with bacilli, that there is a substantial secretion of DNAses, so that there is substantial degradation of any DNA in the medium.

Due to the genetic modification resistant nature of the industrial strains and their prototrophic character which makes them difficult to starve, they show resistance to transformation. It would therefore be of great value to provide for an efficient process for introducing DNA into industrial strains, where the DNA would be stably maintained in the industrial strain, there would be no loss or substantially no loss of viability and activity of the industrial strain and high yields of endogenous and exogenous polypeptide or protein products could be obtained.

Furthermore, selection of cells is difficult where the modification or transformation of the host cells involves increasing the copy number of an endogenous gene or previously introduced gene, where the gene is not involved with survival selection. It is therefore highly desirable to have an efficient process in which the presence of additional genes (increased copy number) can be detected and selected for.

Description of the Prior Art

Genetic manipulations of B. subtilis have been reported by Yoneda et al., Biochem. Biophys. Res. Commun. (1973) 50:765-770; Yoneda and Maruo, J. Bacteriol. (1975) 124: 48-54; Sekiguchi et al., J. Bacteriol. (1975) 121:688-694; Hitotsuyanagi et al., Agric. Biol. Chem. (1979) 43:2342-2349; Yoneda, Appl. Env. Microbiol. (1980) 39:274-276. Successful applications of recombinant DNA technology with respect to production improvements of certain, efficiently transformable laboratory strains of B. subtilis have been reported, e.g., alpha-amylases, beta-lactamases, dihydrofolate reductase, interferon and insulin (Palva, Gene (1982) 19:81-87; Shinomiya et al., Agric. Biol. Chem. (1981) 45:1733-1735; Gray and Chang, J. Bacteriol. (1981) 145:422-428; Williams et al., Gene (1981) 16:199-206; Palva, Gene (1983) 22:229-235). The difficulties in genetically manipulating Bacillus licheniformis soil isolates is reported by Thorne and Stull, J. Bacteriol. (1966) 91:1012-1014. See also, U.K. Patent Application GB2091628; European Patent Application 0 034 470; European Patent Application 0 032 238; and European Patent Application 0 077 109, which disclosure is incorporated herein by reference, as it relates to pUR1523.

SUMMARY OF THE INVENTION

Novel methods and products involving genetically modified unicellular microorganism strains, particularly industrial Bacillus strains, are provided. Extrachromosomal DNA containing a gene of interest capable of expression in an industrial strain host is introduced into an appropriate bacterial host, conveniently a laboratory strain host related

to the industrial strain, and the modified bacterial host combined with an industrial strain under fusing conditions. Cells of the industrial strain containing the gene(s) of interest are selected by means of a marker associated with the gene of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic view of the plasmid pGB33;

Figure 2 is a diagrammatic view of the plasmid pGB34;

Figure 3 is a diagrammatic view of the plasmid pLC28;

Figure 4 is a diagrammatic view of the plasmid pLC83;

Figure 5 is a diagrammatic view of the plasmid pLC87;

Figure 6 is a diagrammatic view of the plasmid pGB35;

Figure 7 is a diagrammatic view of the plasmid pLP33;

Figure 8 is a diagrammatic view of the plasmid pLP87; and

Figure 9 is a SDS polyacrylamide gel-electrophoretogram of products secreted by various Bacillus industrial strains relative to protein molecular weight markers.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods are provided for the genetic manipulation of industrial unicellular microorganism strains for use in fermentation for the production of polypeptide products in good yield. Bacillus strains will be used as paradigmatic of other microorganisms. The resulting modified industrial strains retain the desirable characteristics of industrial strains, while providing enhanced yields of expression products of endogenous (same species) or exogenous (different species) genes.

The method involves introducing extrachromosomal DNA into a bacterial host, which is capable of replicating the DNA and is readily susceptible to the introduction of the extrachromosomal DNA. The modified bacterial host cell containing the extrachromosomal element is then combined with the industrial Bacillus strain under fusing conditions, where the recipient Bacillus cells may be subsequently selected for the presence of the gene or genes of interest originating with the extrachromosomal element.

The subject invention may be divided up into the following parts: (1) preparation of the plasmid construct, including the gene(s) for which enhanced expression in the Bacillus host is desired; (2) cloning of the plasmid construct in a compatible host, which can be used for fusion with the industrial Bacillus strain; (3) fusion of the plasmid construct-containing host with the industrial Bacillus strain, including selection of such industrial strain; and (4) growing of said strain in an appropriate nutrient medium for production of the expression product of the gene(s) of interest.

The gene(s) of interest may be any prokaryotic or eukaryotic gene. These genes may include bacterial genes, unicellular microorganism genes, mammalian genes, or the like. The structural genes may be prepared in a variety of ways, including synthesis, isolation from genomic DNA, preparation from cDNA, or combinations thereof. The various techniques of manipulation of the genes are well-known, and include restriction digestion, resection, ligation, in vitro mutagenesis, primer repair, employing linkers and adapters, and the like. Thus, DNA sequences obtained from a host may be manipulated in a variety of ways, depending upon the requirements of the DNA construction. See Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982.

Where the gene is obtained from a host which has transcriptional and translational initiation and termination regulatory signals which are recognized by the industrial Bacillus strain, it will usually be convenient to maintain the

5'- and 3'-flanking regions of the structural gene to provide a cistron capable of expression in the industrial Bacillus host. The transcriptional initiation region may provide for constitutive or inducible expression, so that in appropriate situations, the host may be grown to high density before high levels of expression of the structural genes of interest are obtained.

Where the structural gene is from a source whose regulatory signals are not recognized by Bacillus, it will be necessary to obtain regulatory regions recognized by Bacillus and insert the structural genes between the initiation and termination regulatory signals. In some instances the exogenous structural gene with its own stop codon(s) may be inserted in reading frame behind the N-terminus codons of an endogenous Bacillus structural gene having its natural regulatory signals. The resulting product may then have some, for example, 0 to 30, additional N-terminal amino acids. Alternatively, operons can be prepared, where a single promoter provides for transcriptional initiation of a messenger RNA which codes for a plurality of polypeptides.

In some instances, it may be desirable that the expression product be secreted. Where the expression product is naturally secreted and the leader signal and processing signal(s) are recognized by the Bacillus host, this will entail no difficulty. However, where the product is not normally secreted, or Bacillus does not recognize the secretory signals and/or processing signal(s), that is the signals are not functional to a satisfactory degree in Bacillus, then it may be necessary to isolate or synthesize DNA sequences coding for the secretory signals and processing signal(s) of a Bacillus polypeptide and join them to the 5'-end of the structural gene in proper reading frame.

The structural genes may express such a variety of polypeptides or proteins, such as enzymes, hormones, lymphokines, surface proteins, blood proteins, structural proteins, immunoglobulins, or the like, from mammals, unicellular microorganisms, e.g., bacteria, fungi, such as yeast, or filamentous fungi, algae, protozoa, etc., plants, or

other DNA source. Of particular interest are enzymes, more particularly hydrolases and more particularly proteases and saccharidases. Illustrative of such enzymes are endopeptidases, exopeptidases, serine and non-serine proteases, alpha- and beta-amylases (particularly thermostable alpha-amylase), and the like.

There are a wide number of vectors which can be employed for the compatible host as well as the Bacillus strain, where the replication systems may be the same or different for the compatible host and the Bacillus strain. (By vector is intended a replication system(s) compatible with one or more hosts, usually a marker for selection in the host and at least one convenient, usually unique, restriction site.) Usually, it will be convenient to have the compatible host a non-industrial or laboratory Bacillus strain, although this is not necessary and in some instances other organisms may be used, such as E. coli. The vector will include one or more replication systems so that it is at least capable of being cloned in the compatible host. The replication system can provide for either high or low copy number, preferably a copy number of at least about 10 and generally not more than about 100.

Depending upon whether one desires integration of the structural genes in the industrial Bacillus strain or maintenance on an extrachromosomal element, a replication system for Bacillus may or may not be included. Alternatively, for integration, one may provide for stretches of homology in the vector or plasmid construct with the Bacillus genome to enhance the probability of recombination.

In addition to the replication system, there will be at least one marker and there may be more than one marker, usually not more than about three markers. By marker is intended a structural gene capable of expression in a host, which provides for survival selection. By "survival selection" is intended imparting prototrophy to an auxothropic host, biocide or viral resistance. For prototrophy, various genes may be employed, such as leu, ura, trp, or the like. For biocide resistance this may include resistance to antibiotics,

e.g. neo, cam, tet, tun, kan, or the like. Other markers include resistance to heavy metals, immunity, and the like. The various DNA sequences may be derived from diverse sources and joined together to provide for a vector which includes one or more convenient, preferably unique, restriction sites to allow for insertion or substitution of the structural genes at such sites or in place of lost fragments to provide the plasmid construct.

Once the plasmid construct has been prepared, it may now be cloned in an appropriate compatible host referred to as the compatible or cloning host. Any host may be used which is convenient, is readily transformable, and allows for replication of the plasmid construct and transfer to the industrial Bacillus strain through fusion. A large number of laboratory strains are available which have a high efficiency of transformation and are usually auxotrophic and/or antibiotic sensitive. The use of a Bacillus host for cloning of the plasmid construct has many advantages in that it permits the use of a single replication system as well as the same marker for survival selection in both the compatible host and the industrial strain. Thus, for the most part, the plasmid construct will be cloned in an appropriate Bacillus host. The Bacillus host need not be the same Bacillus strain as the industrial host and will be chosen primarily for convenience. The plasmid construct may be introduced into the compatible host in accordance with conventional techniques, such as transformation, employing calcium precipitated DNA, conjugation, or other convenient technique. The compatible host may then be grown in an appropriate nutrient medium, under selective conditions to select for a host containing the plasmid construct. For auxotrophic hosts, the nutrient medium is deficient in the required nutrient, while for biocide resistance, a cytotoxic amount of the biocide(s), e.g. antibiotic(s), is employed in the nutrient medium. After growing the compatible host to a sufficient density, the compatible host is then treated to prepare the cells for fusion.

Conveniently, the cells are killed with a cytotoxic agent prior to or during protoplast formation. Various agents

may be employed, including antibiotics, but iodoacetamide is found to be convenient, efficient, and does not interfere with the subsequent fusion. Protoplasts are prepared from the cells in accordance with conventional ways, e.g., lysozyme or zymolyase treatment, and the protoplasts carefully suspended in an appropriate medium having proper osmolality for maintaining the integrity of the protoplast.

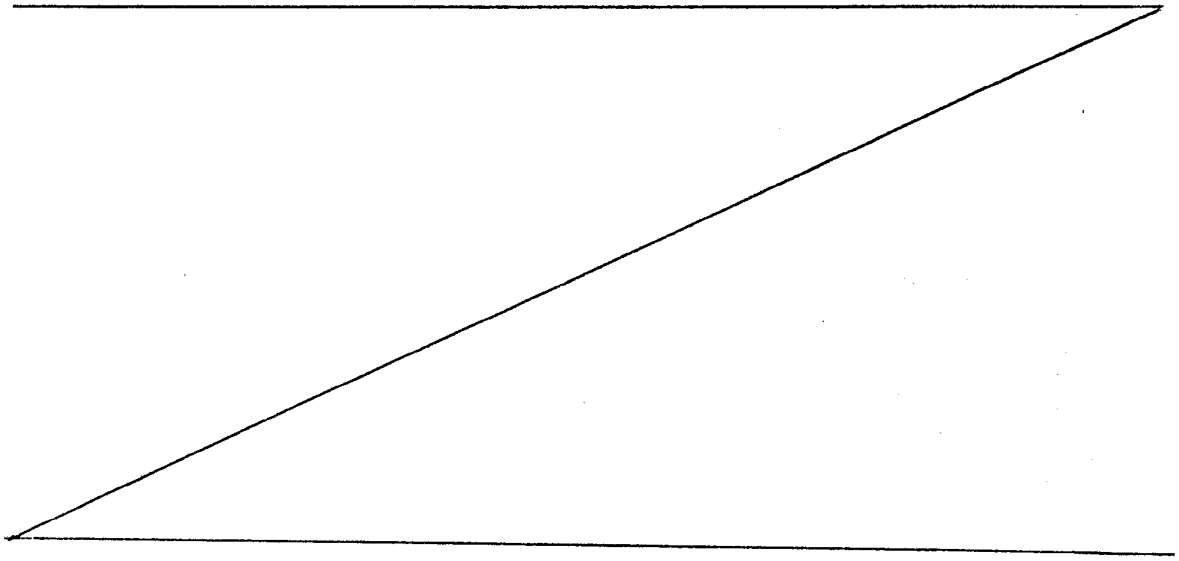
The industrial Bacillus acceptor strain is also treated to form protoplasts in a similar manner as the compatible host strain, but viable cells are employed for preparing protoplasts. Various Bacillus strains may be employed which have the desired traits of an industrial Bacillus strain, such as subtilis, licheniformis, amyloliquefaciens, stearothermophilus and coagulans, preferably licheniformis and subtilis. The industrial Bacillus strains are chosen from organisms which may be isolated in the soil or available from depositories or other sources or obtained by modification of such Bacillus strains. The industrial Bacillus strains are characterized by being resistant to genetic exchange, such as phage infection or transformation. The strains are stable and may or may not be capable of spore formation. They are prototrophic and provide for high yields of endogenous protein products, such as the enzymes alpha-amylase and various proteases. They also are found to secrete DNAses, which results in the degradation of DNA in the medium, providing for protection against genetic exchange.

The dead compatible host protoplast and the viable industrial Bacillus host protoplast are combined in the presence of an appropriate fusogen. While any fusogen may be employed which provides a desired efficiency, for the most part polyethylene glycol is found to provide high efficiency of fusion with great convenience. The ratio of the dead protoplast to the Bacillus acceptor strain will be preferably at least 1:1 and excesses of the dead protoplast may be employed. After a short time, the fusogen mixture is replaced with an appropriate nutrient medium and cells regenerated in a selective medium, conveniently by plating on an agar plate.

The clones may then be screened in appropriate ways for detection of the expression of the additional structural genes. Various techniques may be employed, particularly where enzymes are involved which have well established methods of detection. Alternatively, where enzymes are not involved or there is no available detection system, antibodies, DNA or RNA hybridization, or bioassays can be employed for screening the clones to determine the presence of the plasmid construct and expression of the structural gene(s) of interest.

The industrial Bacillus host containing the plasmid construct or chromosomally integrated plasmid constructs or fragments thereof is then grown in a nutrient medium under conventional fermenting conditions. The fermenting may be continuing until the broth is exhausted. Where the product has been secreted, the product may be isolated from the broth by conventional techniques, e.g., extraction, chromatography, electrophoresis, or the like. Where the product is retained in the cytoplasm, the cells may be harvested by centrifugation, filtration, etc., lysed by mechanical shearing, detergent, lysozyme, or other techniques and the product isolated as described previously. By employing the subject method greatly enhanced yields of endogenous polypeptides can be achieved, usually at least about 150% times the yield of the parent cell, more usually at least 175%, and preferably at least about 200% times the yield of the parent cell.

The following examples are offered by way of illustration and not by way of limitation.



Example I

Isolation of chromosomal DNA

Chromosomal DNA of B. licheniformis industrial strain T5, deposited with Centraal Bureau voor Schimmelcultures, Oosterstraat 1, Baarn, the Netherlands (hereinafter CBS) on July 6, 1983 under No. 470.83, was isolated from 3L cultures, that were grown overnight at 37°C under aeration. Cells were spun down at 10,000rpm for 10min in a Sorvall GSA rotor, suspended in 10ml sucrose-Tris buffer containing 25% w/v sucrose and 50mM Tris-HCl pH 8.0, and lysed by addition of 0.5ml lysozyme solution (20mg/ml) and incubation for 15min at 37°C. After addition of 2ml EDTA (0.5 M) and incubation for 5min at 0°C, 1ml 20% (w/v) sodium dodecyl sulphate (SDS) was added. The suspension was then extracted with a 1:1 phenol-chloroform mixture. The supernatant was separated and carefully overlayed by 2 volumes of pure ethanol after which the DNA was isolated with the aid of a glass rod. After dissolving in distilled water containing 10µg/ml ribonuclease, the DNA suspension was extracted with 1:1 phenol-chloroform, precipitated with 2 volumes of ethanol and resuspended in TE buffer (i.e., 10mM Tris-HCl, pH8.0 containing 1mM EDTA).

Example II

Isolation of plasmid DNA

B. subtilis strain 1G 20, containing plasmid pUB110 (cf. European Patent Specification 0 021 468), was grown overnight in 1L penassay broth medium to which 5µl/ml neomycin had been added. After centrifuging for 15min at 5000rpm in a Sorvall model GSA rotor and resuspending in 15ml sucrose-Tris buffer, the cells were lysed and treated with EDTA and SDS as described in Example I. After addition of NaCl to a final concentration of 1M the supernatant was stored overnight at 4°C and then centrifuged for 45min at 12500rpm in a Sorvall type SS 34 rotor. The upper 70% (v/v) of the supernatant was

treated with 20µg/ml DNAse-free RNAse (for 0.5h at 37°C), and extracted with phenol-chloroform (1:1) mixture, followed by extraction by chloroform alone.

The DNA was precipitated from the extracted supernatant by addition of 0.2 volume of 5M NaCl and 0.25 volume of 40% (w/v) polyethylene glycol 6000, followed by incubation for 16h at 4°C. After precipitation and centrifugation (30min at 12500rpm, Sorvall type SS 34 rotor) the DNA was resuspended in 2-3ml TE buffer (as in Example I) and the dispersion made pH12.0 with 4M NaOH. The pH was then adjusted to 8.5 and the suspension was extracted with phenol. After precipitation of the extract with ethanol the plasmid DNA was resuspended in a small volume of TE buffer.

Plasmid pUR1523 (cf. European specification A-77109) DNA from E. coli was isolated according to the method described by Birnboim and Doly, Nucl. Acids Res. (1979) 7: 1513-1523.

Example III

a) Construction of the alpha-amylase containing recombinant plasmids pGB33 and pGB34 (Figs. 1 and 2)

5µg chromosomal DNA, isolated from the Bacillus production strain T5 (as described in Example I) and 1µg pUB 110, isolated from B. subtilis 1G 20 (as described in Example II) were digested with EcoRI. After thermal denaturation of the restriction enzyme for 7.5min at 65°C, the DNA was precipitated with ethanol and resuspended in 20µl of a ligase mixture containing 20mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 10mM dithiothreitol (DTT), 0.2mg/ml bovine serum albumin, 0.5mM ATP and 1 unit of T_4 ligase (Boehringer-Mannheim). The mixture was ligated overnight at 4°C. The ligated mixture was transferred into B. subtilis as described in Example IV below. Plasmid DNA was isolated (using the method described in Example II) from selected recombinant microorganisms and analysed with restriction endonucleases. Plasmid pGB33 was a recombinant of pUB110 and a chromosomal EcoRI fragment of approx. 3kbp,

containing the alpha-amylase cistron. Digesting pGB33 with the restriction endonucleases HindIII and BamHI, followed by S_1 exonuclease resection and ligation with T_4 yielded pGB34 (Fig. 2), which still harbors the alpha-amylase cistron but lacks a DNA segment containing many inconvenient restriction sites. The plasmids pGB33 in B. subtilis 1-85 (= trp^-) and pGB34 in B. subtilis 1S-53 (Bacillus Genetic Stock Center, Ohio, U.S.A.) were deposited with CBS on July 6, 1983 as Nos. 466.83 and 467.83, respectively.

b) Construction of the chymosin containing recombinant plasmids pLC28, pLC83 and pLC87 (Figs. 3, 4 and 5).

0.5µg pGB34 DNA and 0.5µg pUR1523, an E. coli plasmid that harbors the gene for bovine chymosin, were digested with PstI. After thermal denaturation and ligation as described in Example IIIa, the mixture was used for transformation. Plasmid DNA isolated from selected transformants (using the selection procedure described in Example IV below) was analysed with restriction endonucleases. The selected plasmid, called pLC28, was a recombinant of the chymosin gene containing PstI fragment of pUR1523, inserted into the unique PstI site of pGB34 (Fig. 3). Cutting pLC28 with the restriction endonuclease ClaI, followed by resection with the exonuclease Bal31 and ligation with T_4 ligase, yielded pLC83 (Fig. 4), which contains the gene for chymosin, but no longer contains a DNA segment with many inconvenient restriction sites. The plasmids pLC28 and pLC83, both in B. subtilis 1S-53, were deposited with CBS on July 6, 1983 as Nos. 469.83 and 468.83, respectively.

In order to place the chymosin gene in phase behind the alpha-amylase transcriptional and translational initiation regulatory sequences, pLC83 was digested with PstI, resected with nuclease S_1 and ligated with T_4 ligase. The plasmid obtained was called pLC87, and shown to have the correct placement and orientation by sequence analysis. See Figure 5.

c) Construction of the protease containing recombinant plasmids pLP33 and pLP87 (Figures 7 and 8)

100µg chromosomal DNA of the protease producing strain B. subtilis 168 (Bacillus Genetic Stock Center, Columbus, Ohio) was partially digested with the restriction enzyme ClaI. After phenol extraction the DNA fragments were separated on a 1% agarose gel. By means of electroelution several DNA fractions were eluted from the gel and ligated with ClaI linearized pGB35 (a recombinant plasmid containing the Bacillus vector pGL112, W.M. de Vos, Thesis University of Groningen 1983, and the alpha-amylase gene of pGB33, see Fig. 6). The ligation products were transferred into competent B. subtilis RUB331 cells in the manner as described in Example IV. The chloramphenicol-resistant transformants obtained were analysed for enhanced protease activity ($prot^+$) and reduced alpha-amylase production capacity (amy^-) (see Example IV) on 0.4% casein-amylose plates. Plasmid DNA was isolated from $prot^+amy^-$ transformants and restriction site mapped; pLP33 appears to contain a 3.3 kbp ClaI fragment (Fig. 7) which includes a structural gene coding for a serine protease. pLP87 (Fig. 8) has a chromosomal ClaI insert of 1.8 kbp containing the genetical information for a non-serine protease.

Example IV

Transformation of Bacillus strains

B. subtilis 1-85 (trp^-amy^-) transformed by incubating 1ml cell suspension and 1µg ligated DNA for 0.5h at 37°C with gentle shaking (Anagnostopoulos and Spizizen, J. Bacteriol. (1961) 81:741-746). Transformants were selected for antibiotic resistance on minimum medium agar plates, to which 0.02% (w/v) casamino acids (Difco) and 10µg/ml of an antibiotic were added. These transformants were then analysed for the presence of the desired structural gene.

In the case of alpha-amylase this was performed by looking for halos after covering the plates with a solution

containing 0.6% (w/v) KI and 0.3% (w/v) I_2; by positive hybridization to a ^{32}P labeled DNA probe synthesized in vitro in accordance with the N-terminal amino acid sequence of the biologically active enzyme; by immunoprecipitation with antibodies against the enzyme and by comparative isoelectrofocusing.

In the case of chymosin, positive selection was carried out via hybridization with ^{32}P labeled pUR1523 DNA and by immuno-precipitation with anti-chymosin antibodies.

To identify the gene for a Bacillus protease the transformants were tested for their ability to form halos on casein minimal medium agar plates. The difference between serine and non-serine protease was determined by the method of Scheiner and Quigley (Anal. Biochemistry (1982) 122:58-69).

The selected transformants were used as donor strains in cell fusion experiments.

Example V

Cell fusion and regeneration

The transformed B. subtilis strain (B. subtilis strain 1-85 containing pGB33, B. subtilis strain 1S-53 containing pLC87 or B. subtilis strain RUB331 containing pLP33) was grown overnight in 50ml NBSG-X medium (Thorne and Stull, J. Bacteriol. (1966) 91:1012-1020 (Table II, page 1014)) with a relevant antibiotic therein at 37°C. The cultures were diluted 1:1 with fresh NBSG-X medium and grown for another 1-1.5 h in the presence of 10mM iodoacetamide. After centrifuging for 10min at 5000rpm in a Sorvall type GSA rotor and resuspending in 10ml SMM buffer containing 1.5M sucrose, 0.06M $MgCl_2$ and 0.06M maleate, the cells were protoplasted by incubating the cells for 2h at 37°C in the presence of 2mg/ml lysozyme. The protoplasts were spun down (10min x 5000rpm), resuspended in 5ml SMML buffer (L-broth in which 1.5M sucrose, 0.06M $MgCl_2$ and 0.006M maleate has been dissolved), mixed and repelleted. After being resuspended, the protoplasts were mixed with the protoplasts of the acceptor strain T5,

viable cells protoplasted as described above, and incubated for 2min in the presence of approximately 30% (w/v) of polyethylene glycol 6000. After 1:3 dilution with SMML medium and centrifugation, the pellet was resuspended in a small volume of SMML medium. Then 100µl aliquots were plated on regeneration agar plates containing 0.7% (w/v) K_2HPO_4, 0.3% (w/v) KH_2PO_4, 0.125% (w/v) $(NH_4)_2SO_4$, 0.035% (w/v) $MgSO_4.7H_2O$, 1.5% (w/v) agar, 3.7% (w/v) KCl, 0.1% (w/v) glucose, 0.01% (w/v) bovine serum albumin supplemented with 0.1% (w/v) spore solution containing 0.2% (w/v) $MnSO_4$, 0.2% (w/v) $ZnSO_4$, 0.2% (w/v) $CoCl_2$, 0.5% (w/v) $FeSO_4$, 6% (w/v) NaCl and 0.5% (w/v) $CaCl_2$. Moreover these plates contained the relevant antibiotic, in the case of pGB33, pLP33 and pLC87 100-160µg/ml neomycin. After incubation at 37°C for at least 72h, the plates were replica plated on heart-infusion agar plates, containing also another antibiotic to which the acceptor strain is resistant but to which the donor strain is sensitive. Fusants designated as Type A were analysed according to the methods described in Example IV. In the case of alpha-amylase the procedure was repeated as follows. Type A fusants were fused with B. subtilis protoplasts containing pGB36 (a recombinant plasmid of pTL12, containing the gene that encodes resistance for trimethoprim (Tanaka and Kawano, Gene (1980) 10:131-136) and the EcoRI restriction fragment of pGB33, containing the gene that codes for B. licheniformis alpha-amylase), yielding fusants designated as Type B. Both Type A and Type B fusants were characterized by genomic analysis using ^{32}P labeled plasmid pGB33 and by comparative SDS gel-electrophoresis. Finally the fusants obtained were selected for the fermentative production of the various enzymes.

Example VI

Fermentative production of alpha-amylase, protease and chymosin by genetically engineered Bacillus production strains.

The genetically engineered production strain of B. licheniformis T5, obtained by fusion with the B. subtilis strain 1-85, containing pGB33 (as described in Example V), was cultivated for 7 days in an industrial nutrient broth under such fermentation conditions that the production of secreted protein is at least 0.5% w/v, alpha-amylase being the major constituent. The production of this engineered strain was compared to the production of the parental strain B. licheniformis T5. As evidenced by an SDS polyacrylamide gel-electrophoretogram of products secreted by the production strain B. licheniformis T5 (see Fig. 9, lane 3) and those secreted by the genetically engineered strain B. licheniformis T5, containing pGB33 (see Fig. 9, lane 2), the production of alpha-amylase was significantly increased by the introduction of the plasmid pGB33.

Comparable results were obtained with the plasmid pLP33, which introduction improved the production of protease and with plasmid pLC87 which introduction resulted in a B. licheniformis T5 strain, which is capable of producing chymosin.

Table I shows the summarized results of a quantitation of the improvements of the respective genetically engineered microorganisms.

Table I

Organism	plasmid	alpha-amylase prod.
B. licheniformis T5	-	100% *
B. licheniformis T5	pGB 33	180% **
B. lich. T5(TypeA)	pGB 36	230% ***
Organism	**plasmid**	**protease production**
B. licheniformis T5	-	100%
B. licheniformis T5	pLP 33	145%
Organism	**plasmid**	**chymosin production**
B. licheniformis T5	-	-
B. licheniformis T5	pLC 87	+

* 1 alpha-amylase gene;
** 2 alpha-amylase genes;
*** 3 alpha-amylase genes.

The subject method has shown itself as highly successful with Bacillus. However, many unicellular microorganisms other than Bacillus find use in industrial fermentation and have properties such as the industrial Bacillus strains which make them refractory to efficient transformation. Therefore, the subject method could find application with industrial strains of other prokaryotic and eukaryotic organisms, such as other bacteria, fungi, e.g., yeast and filamentous fungi, protozoa, algae, etc. Species of genera, such as Aspergillus, Candida, Escherichia, Kluyveromyces, Penicillium, Pseudomonas, Saccharomyces and Streptomyces, are of particular interest.

Of particular interest in these organisms and in part as indicated for Bacillus is the industrial production of endogenous polypeptides, such as alpha-amylases, amyloglucosidases, catalases, cellulases, chymosins, beta-galactosidases, glucose isomerases, hemicellulases, invertases, lactases, lipases, pectinases, pectin esterases, penicillinamidases, penicillinases, proteases, exo- and endopeptidases, pullulanases and xylanases. Also of interest are exogenous proteins, such as mammalian blood proteins, e.g., Factor VIII, both C and R, serum albumin, tissue plasminogen activator, other blood Factors, e.g., V, VI, VII, IX, X, XI and XII, lymphokines, interferons, e.g., alpha-, beta- and gamma-, mammalian enzymes, cell surface receptors, immunoglobulins, etc.

It is evident from the above results that a simple and efficient procedure is provided for stably introducing homologous or heterologous genes into industrial Bacillus strains, while retaining the desirable characteristics of the strains and providing for the enhanced competence of the cells in the increased production of a desired expression product endogenous to the Bacillus host or the production of a novel expression product of interest. High efficiencies of transfer are achieved and whee substantial homology exists between the plasmid construct and the industrial Bacillus strain chromosome, single or multiple copy integration of the structural genes may be achieved. The subject method greatly enhances the capability of modifying presently existing industrial Bacillus strains to provide efficient fermentative production of a wide variety of polypeptides and proteins of interest.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

CLAIMS

1. A method for efficiently introducing into an unicellular microorganism strain host DNA capable of stable maintenance and expression of a polypeptide of interest in said unicellular microorganism strain host, said method comprising: combining in the presence of a fusogen, first readily transformable host cells containing said DNA as an extrachromosomal element capable of maintenance in said first host and said unicellular microorganism strain host cells under conditions providing for selection of said strain host cells, whereby said DNA is transferred to said strain host cells; and selecting unicellular microorganism strain host cells containing said DNA.

2. A method according to Claim 1, wherein the unicellular microorganism strain host is an industrial strain host.

3. A method according to Claim 2, wherein the industrial strain host is resistent to genetic modification by a vector.

4. A method according to Claim 3, wherein the vector is a plasmid, a phage or a cosmid.

5. A method according to Claim 2, wherein the industrial strain host has a production level of at least 0.5% w/v secreted protein under industrial fermentation conditions.

6. A method according to claim 1 for producing a microorganism having increased ability to produce a desired protein or polypeptide, said method comprising:

(a) fragmenting DNA containing a gene coding for the desired protein or polypeptide
(b) ligating a vector to said fragmented DNA
(c) transforming auxiliary cells with said ligated vector
(d) selecting the transformed cells for the presence and

optionally the expression of the desired gene and optionally inactivating said transformed auxiliary cells

(e) fusing protoplasts of cells from a selected host microorganism and said optionally inactivated transformed auxiliary cells, and

(f) selecting the fused regenerated cells containing the gene coding for the desired protein or polypeptide and having increased ability to produce the desired protein or polypeptide.

7. The method of claim 6 wherein the DNA containing a gene coding for the desired protein or polypeptide is fragmented by one or more restriction enzymes.

8. The method of claim 1 wherein the unicellular microorganism strain host is selected from the group consisting of bacteria, yeasts and fungi.

9. A method according to claim 1 for efficiently introducing into an industrial Bacillus strain host DNA capable of stable maintenance and expression of a polypeptide of interest in said industrial Bacillus strain host, said method comprising:

combining in the presence of a fusogen, first readily transformable prokaryotic host cells containing said DNA as an extrachromosomal element capable of maintenance in said first host and said industrial Bacillus strain host cells under conditions providing for selection of said industrial Bacillus strain host cells, whereby said DNA is transferred to said industrial Bacillus strain host cells; and selecting industrial Bacillus strain host cells containing said DNA.

10. A method according to claim 9, wherein said industrial Bacillus strain is the strain licheniformis.

11. A method according to claim 9, wherein said polypeptide is an endogenous enzyme of said Bacillus strain.

12. A method according to claim 11, wherein said endogenous enzyme is an alpha-amylase, preferably a thermostable alpha-amylase, or a protease.

13. A method according to claim 1, wherein said conditions providing for selection of said unicellular microorganism strain is employing first killed prokaryotic host cells.

14. A method for efficiently introducing into an industrial Bacillus strain host DNA capable of stable maintenance and expression of a polypeptide of interest in said industrial Bacillus strain host, said method comprising: combining in the presence of a fusogen first killed Bacillus host cells readily capable of transformation and containing said DNA in a plasmid construct having a replication system capable of functioning in Bacillus and said industrial Bacillus strain host cells, where said cells are present as protoplasts, and whereby said DNA is transferred to said industrial Bacillus strain host cells; and selecting industrial Bacillus strain host cells containing said DNA.

15. A method according to claim 14, wherein said commercial Bacillus strain host is the strain licheniformis.

16. A method according to claim 14, wherein said polypeptide of interest is a Bacillus endogenous enzyme.

17. A method according to claim 16, wherein said DNA becomes integrated into the chromosome of said industrial Bacillus strain host.

18. A method according to claim 16, wherein said enzyme is an alpha-amylase, preferably a thermostable alpha-amylase or a protease.

19. A method for producing a polypeptide of interest in an industrial Bacillus strain which comprises:

growing industrial Bacillus strain host cells prepared according to Claim 9; and isolating the polypeptide expressed by said DNA.

20. A method according to claim 19, wherein said polypeptide is secreted by said industrial Bacillus host strain cells; and said isolating is separating said polypeptide from said nutrient medium.

21. A method according to claim 20, wherein said secreted polypeptide is an alpha-amylase, preferably a thermostable alpha-amylase or a protease.

22. An industrial Bacillus host strain cell produced in accordance with the method of claim 12 and capable of producing at least 150% of the amount of alpha-amylase based on the amount of alpha-amylase produced by the parent cell or at least 150% of the amount of a protease based on the amount of said protease produced by the parent cell, respectively.

23. An industrial Bacillus strain cell according to claim 22, wherein said strain is licheniformis.

24. The method of claim 6 wherein the ligated product of step (b) is a plasmid selected from the group consisting of pGB33, pGB34, pLC28, pLC83 and pLC87.

25. A plasmid selected from the group consisting of the plasmid pGB33 deposited with CBS under No. 466.83, the plasmid pGB34 deposited with CBS under No. 467.83, the plasmid pLC28 deposited with CBS under No. 469.83 and the plasmid pLC83 deposited with CBS under No. 468.83.

26. A genetically engineered microorganism having increased ability to produce a desired protein or polypeptide, obtained by the method of any one of claims 1-24.

27. The genetically engineered microorganism of

claim 18 which comprises a plasmid selected from the group consisting of pGB33, pGB34, pLC28, pLC83 and pLC87.

28. A genetically engineered microorganism of the genus Bacillus containing inserted DNA which microorganism is formed by protoplast fusion of cells of a Bacillus host microorganism which is resistant to genetic modification by a vector and cells of an optionally inactivated auxiliary strain which is capable of uptaking genetic information and in which the desired genetic information has been previously inserted.

29. The genetically engineered microorganism of claim 28 which produces alpha-amylase and which contains at least two stable genes coding for alpha-amylase.

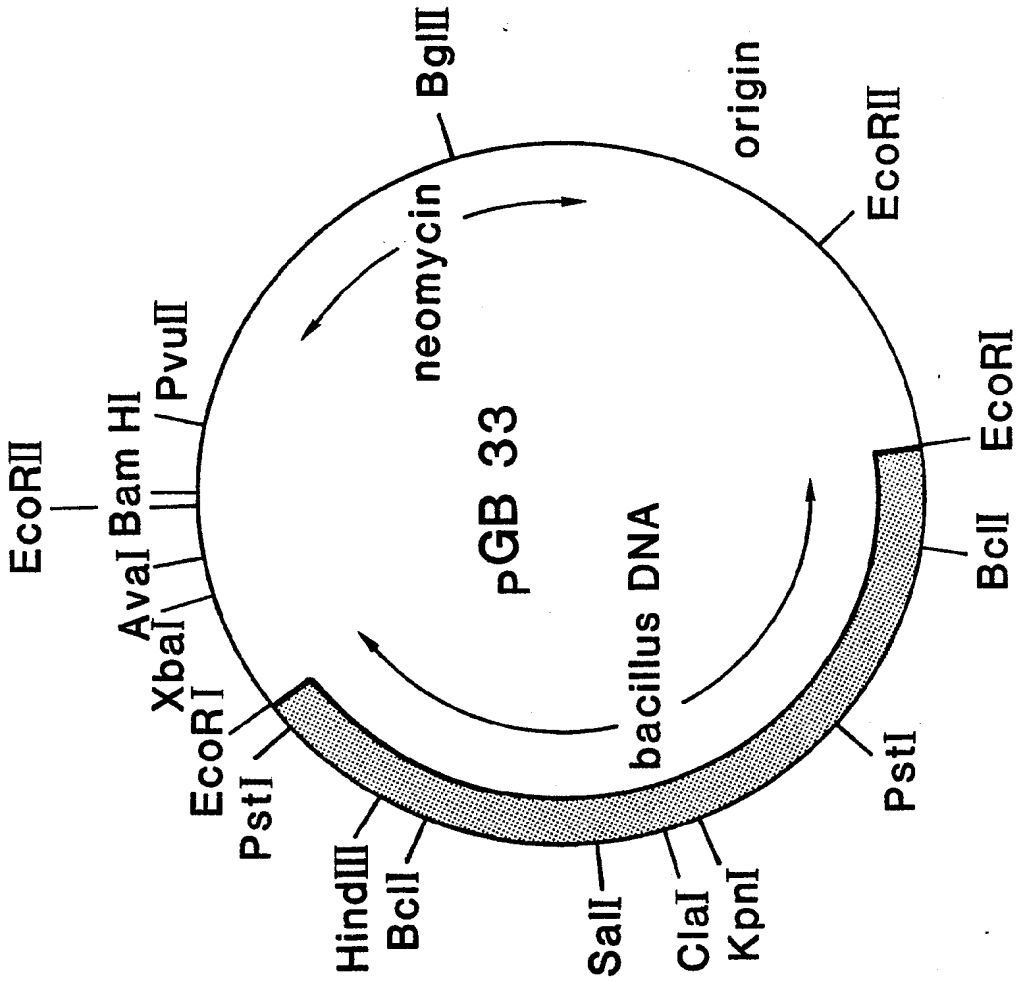
30. Use of a genetically engineered microorganism as defined in any one of claims 26-29, for the production of a protein or polypeptide.

31. A method according to claim 14, wherein said polypeptide of interest is a Bacillus exogenous enzyme.

32. A method according to claim 31, wherein said DNA becomes integrated into the chromosome of said industrial Bacillus strain host.

33. A method according to claim 31, wherein said enzyme is a chymosin.

Fig.1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AvaI
Bam HI
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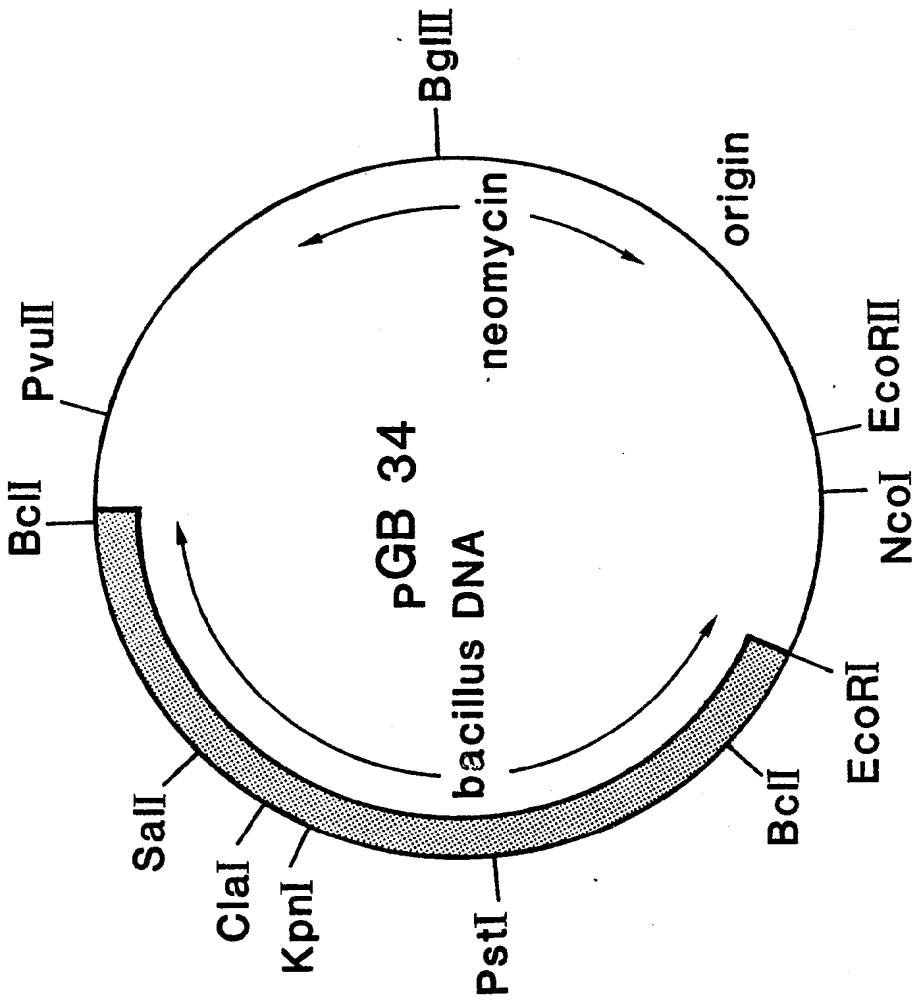
EcoRII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neomycin
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ClaI
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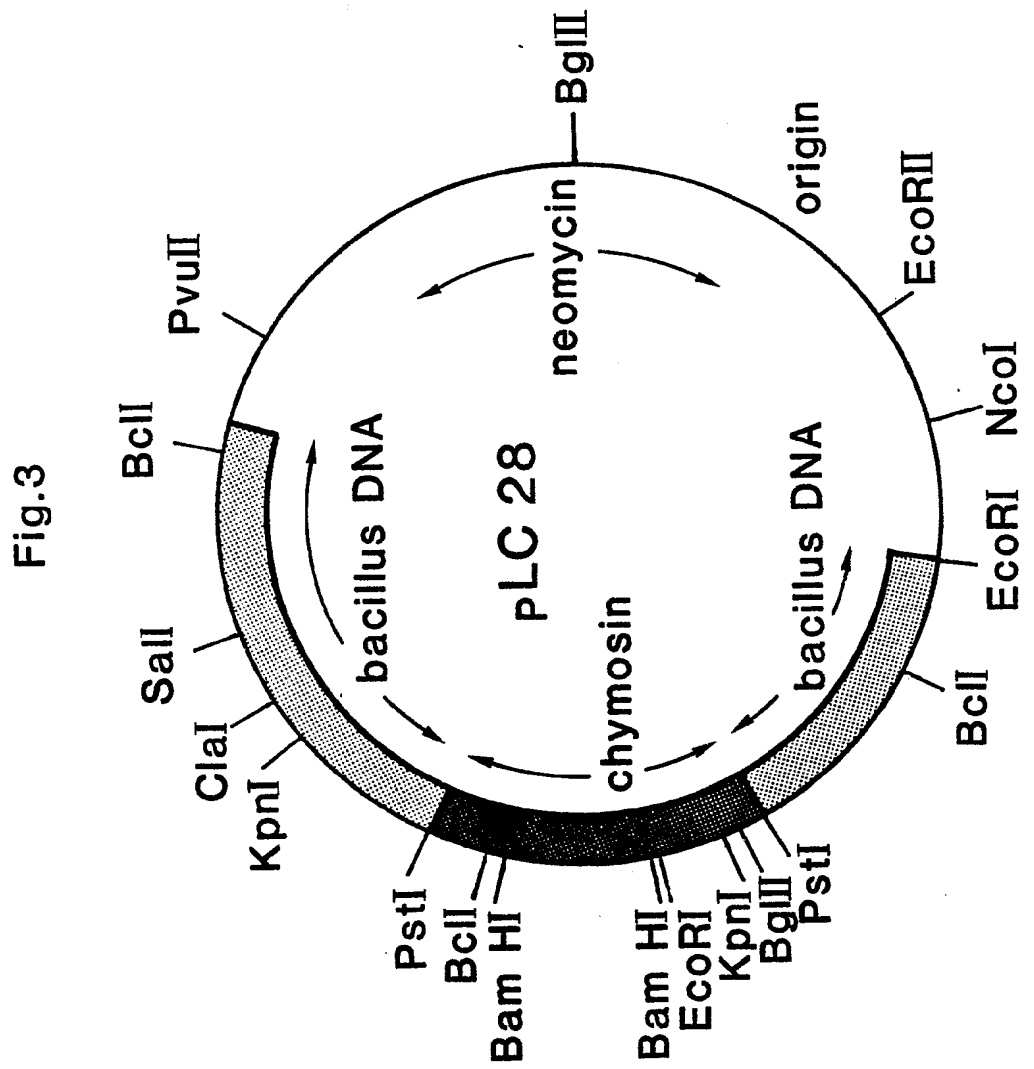
pGB 34
bacillus DNA

Fig.2

Fig.3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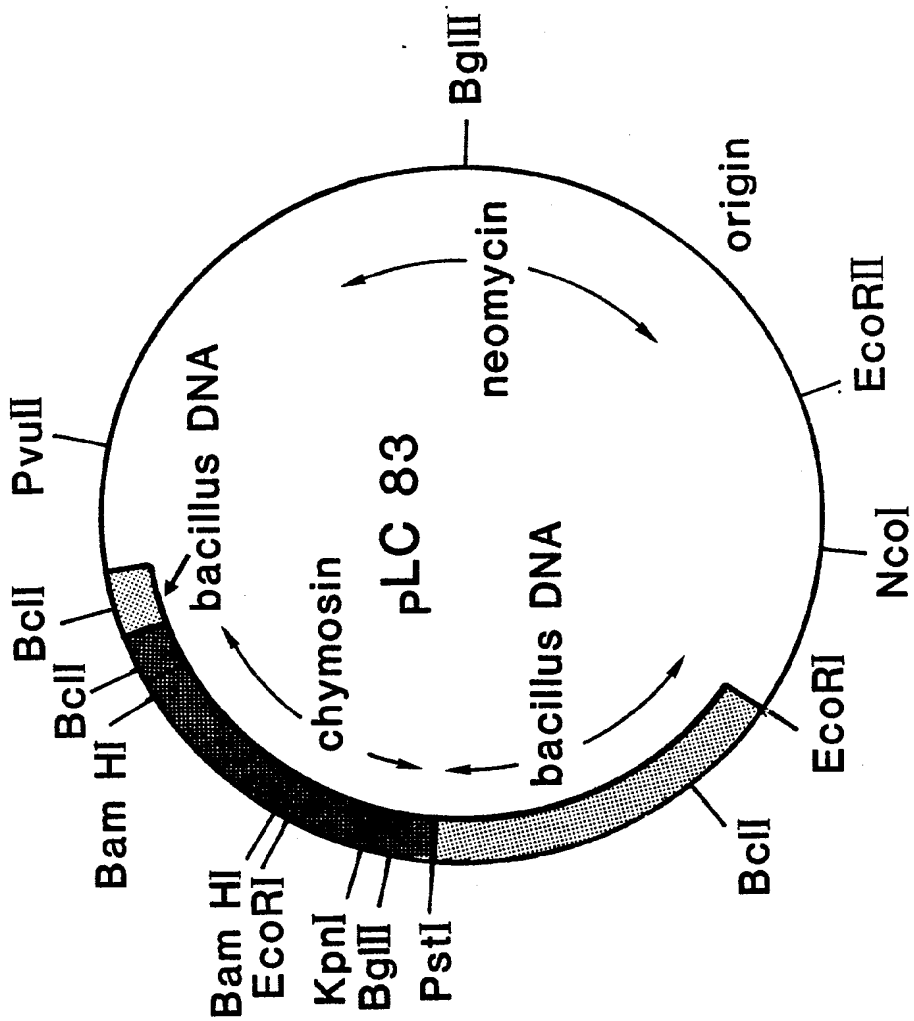
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Bam HI
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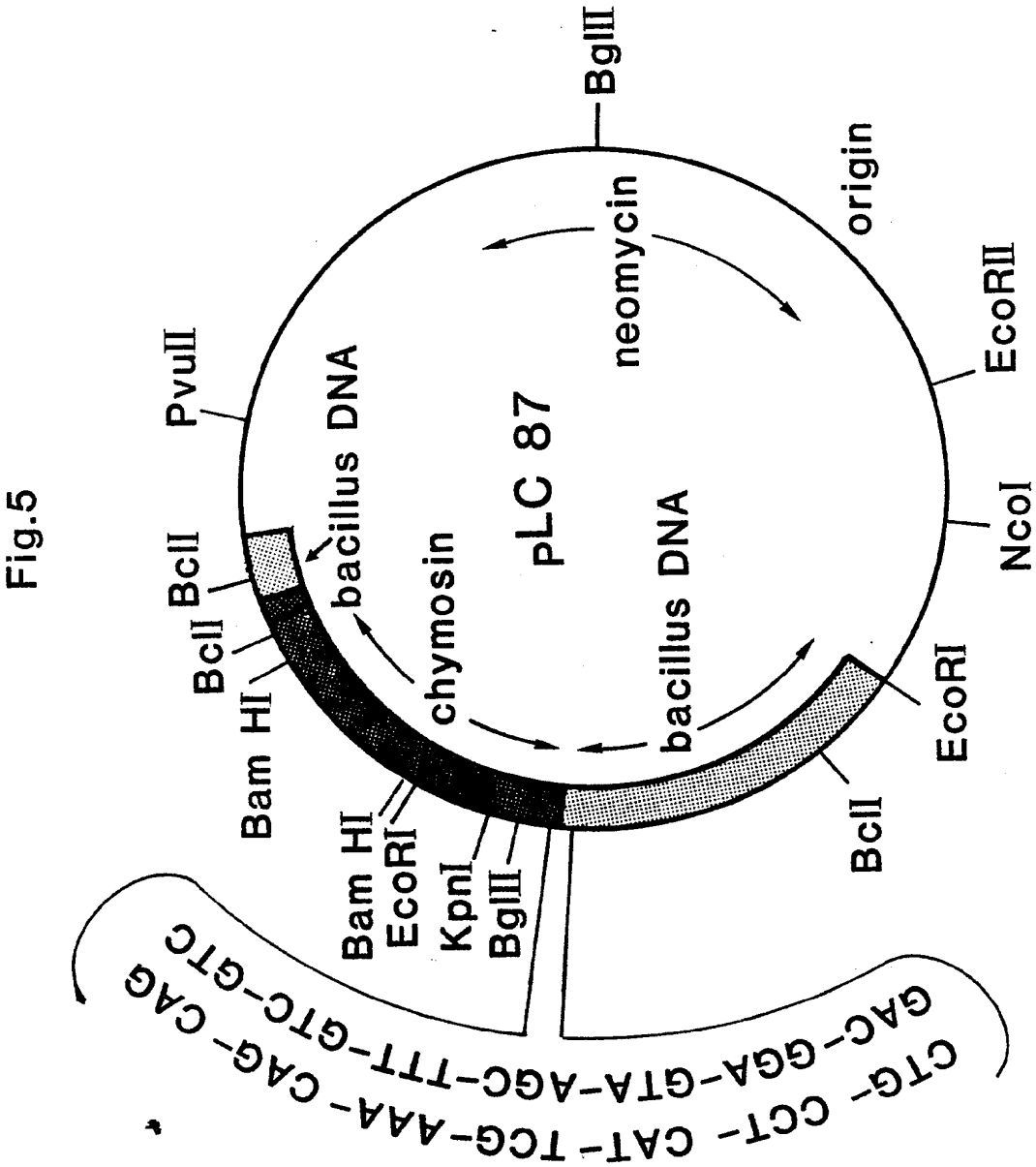
KpnI
BglII
PstI

Fig.4

Fig.5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Bam HI
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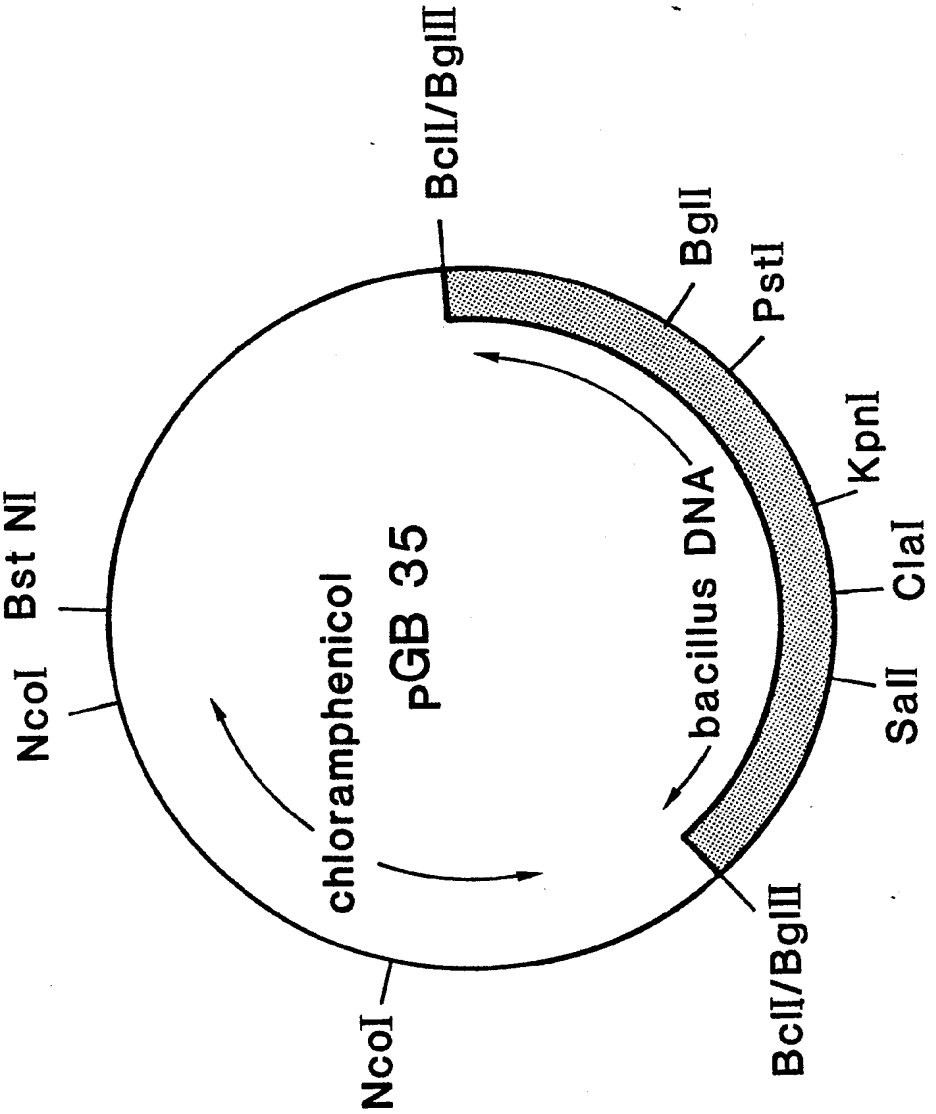
BglII
Bam HI
BclI
PvuII
bacillus DNA
chymosin
pLC 87
neomycin
bacillus DNA
CTG- CCT- CAT- TCG-AAA- CAG- CAG
GAC-GGA-GTA-AGC-TTT-GTC-GTC

Fig.6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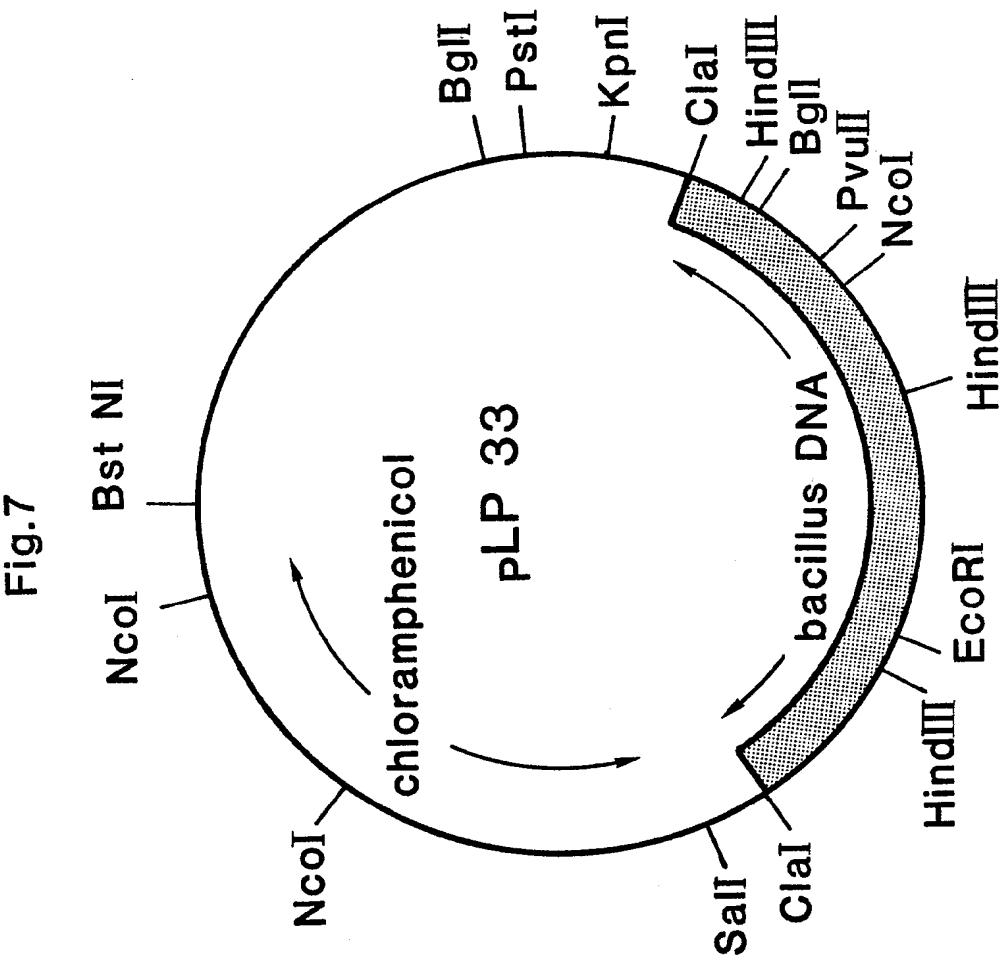
PstI
KpnI
ClaI
SalI
BclI/BglII
NcoI
chloramphenicol
pGB 35
bacillus DNA
NcoI
Bst NI

7/9

Fig.7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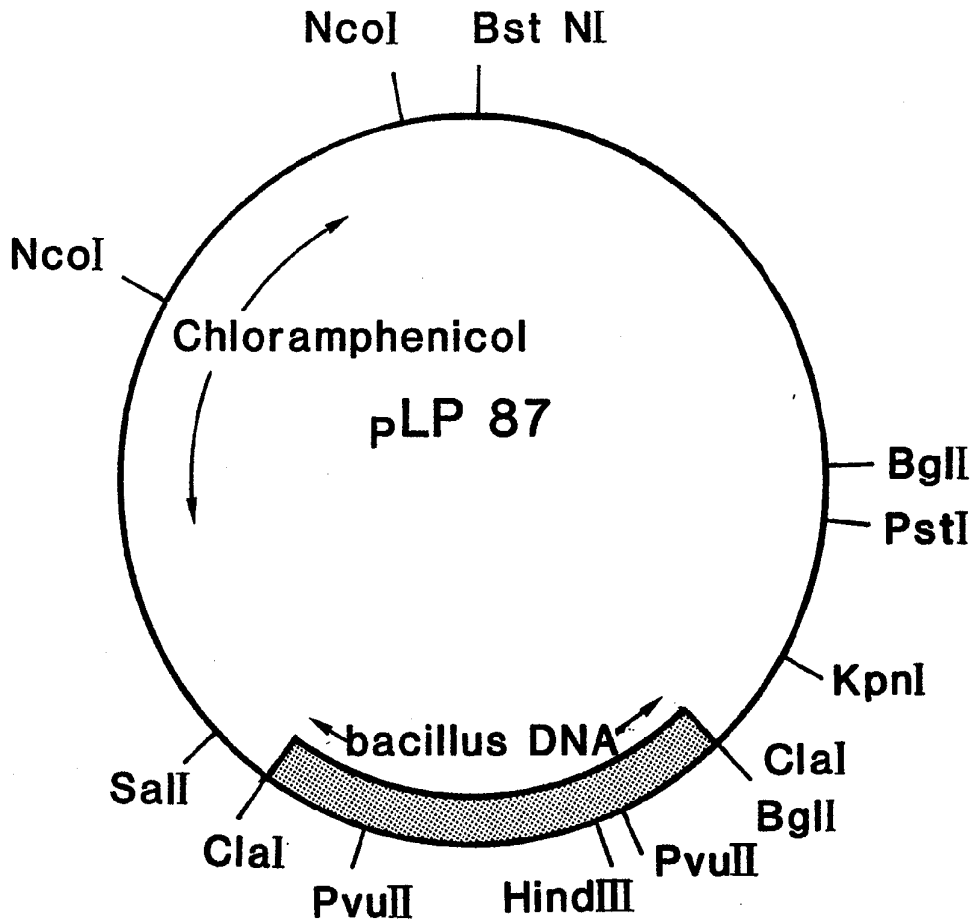
pLP 33
bacillus DNA
NcoI
Bst NI

Fig.8



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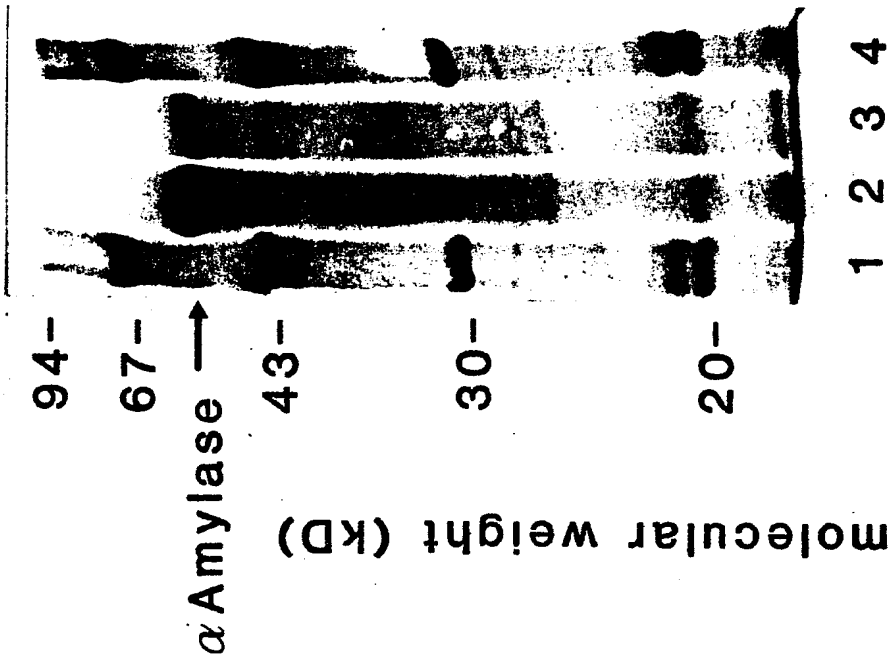

fig. 9

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0134048

. Application number

EP 84 20 0990

EUROPEAN SEARCH REPORT

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y	BIOLOGICAL ABSTRACTS, vol. 73, no. 8, 1982, no. 53368, Biosciences Information Service, Philadelphia, USA; X. JIANG et al.: "The transfer of plasmids through cell fusion in Bacillus subtilis" & ACTA GENET SIN 8(1), 1-7, 1981 * Abstract * ---	1-24, 26-33	C 12 N 15/00 C 12 N 9/28 C 12 N 9/56 // C 12 N 9/64
Y	BIOLOGICAL ABSTRACTS, vol. 73, no. 6, 1982, no. 38805, Biosciences Information Service, Philadelphia, USA; L. MINGFENG et al.: "A study on plasmid pUB110 transfer by Bacillus subtilis protoplast fusion" & ACTA GENET SIN 8(2), 109-115, 1981 * Abstract * ---	1-24, 26-33	
Y	CHEMICAL ABSTRACTS, vol. 96, no. 25, 21st June 1982, page 401, no. 214136e, Columbus, Ohio, USA; J.C. GROSCH et al.: "Transformation of Bacillus licheniformis and Bacillus amyloliquefaciens protoplasts by plasmid DNA" & GENET. CELL. TECHNOL. 1982, 1(GENET. EXCH.), 97-105 * Abstract * --- -/-	1-5,9, 10,14, 15,17-23	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	25-10-1984	DESCAMPS J.A.

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. Application number

EP 84 20 0990

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y	AGRIC. BIOL. CHEM., vol. 47, no. 1, 1983, pages 159-161; Y. TAKEICHI et al.: "Cloning of Bacillus subtilis alpha-amylase structural gene in plasmid pUB110" * Whole document *	1-32	
Y	JOURNAL OF BACTERIOLOGY, vol. 154, no. 2, May 1983, pages 831-837, American Society for Microbiology, USA; M. FUJII et al.: "Molecular cloning of a thermostable neutral protease gene from Bacillus stearothermophilus in a vector plasmid and its expression in Bacillus stearothermophilus and Bacillus subtilis" * Whole document *	12,18, 21,24, 25,27	
D,Y	GB-A-2 091 268 (ILLKA PALVA) * Whole document *	1-33	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
D,Y	EP-A-0 077 109 (UNILEVER N.V.) * Whole document *	24,25, 27,33	
D,Y	EP-A-0 032 238 (THE UNIVERSITY OF ROCHESTER) * Page 8, lines 1-13; example 1 *	11,16, 22	

-/-

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,Y	EP-A-0 034 470 (CPC INTERNATIONAL INC.) * Example IIA; claims *	1-32	
Y	EP-A-0 060 663 (SHIONOGI & CO.) * Whole document *	1-32	
Y	EP-A-0 036 259 (CETUS CORP.) * Whole document *	1-32	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

The present search report has been drawn up for all claims

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